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BIO-CHEMICAL CHARACTERIZATION OF DETOXIFYING ENZYMES IN DDT RESISTANT FIELD ISOLATES OF *PHLEBOTOMUS ARGENTIPES* IN BIHAR, INDIA

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ABSTRACT

In the Indian subcontinent,Visceral Leishmaniasis (VL) is transmitted through the established vector *Phlebotomus argentipes*. There are several reports on development of DDT resistance, the insecticide of choice to *P. argentipes*, based on bio-assay test. In this study, metabolic based mechanisms for DDT resistance were investigated in *P. argentipes* to facilitate the development of novel strategy to control resistance development in the vector. Wild sand flies collected from Baizalpur (District-Sonepur), Chandi (District-Nalanda) and Khusroopur (District-Patna) of Bihar, India were reared for F1 progeny out of which one sub sample was stored at -80^{0}C for biochemical assay while the other was used for insecticide bio-assay test. Biochemical assay was conducted for detoxifying enzymes (Glutathione S-transferase (GST), Acetylcholinestrase (AChE), Cytochrome p450 (Cyt p450) and non- specific esterase (ETS). The total protein pooled from elevated GST samples were also separated on one dimension gel electrophoresis. *P. argentipes* population from Baizalpur, Chandi and Khusroopur villages were found highly resistant to DDT. F1 progeny of *P. argentipes* showed elevated GST in 60% population. There were no elevation in AChE, Cyt p450 and ETS activity. Furthermore, the GST elevation was also observed in 12% SDS-PAGE at around 42 kDa and was confirmed through western blotting. This study provides preliminary biochemical evidence of GST enzyme elevation in DDT resistant Sand fly population, which probably resulted from Malaria and Kala-azar control activities in the area.

KEYWORDS: Bio-Chemical Assay, DDT Resistance, Glutathione S-Transferase Enzyme, Kala-Azar, Metabolic Resistance, Sand Fly

INTRODUCTION

Phlebotomus argentipes (Diptera: Psychodidae) is the established vector for the *Leishmania donovani*, the causative agent of Visceral Leishmaniasis in the Indian Subcontinent. It impedes socio-economic development in the poorest community in India. The overall global burden of leishmaniasis is estimated over 1.3 million cases/year, with a health impact of 2.35 million disability-adjusted life years (DALYs) (WHO, 2002). DDT is being used in vector control since 1960's to control vectors of Malaria. Sand flies were controlled as a collateral benefit. Now days it is separately being used in Kala-azar control programme. There are reports of development of resistance against DDT in some parts of

Bihar to *P. argentipes* based on cone/tube bio-assay test (Singh et al, 2001; Dinesh et al., 2010). The development of resistance against DDT in *P. argentipes* is a major problem associated with the disease control (Kishore et al, 2006). It is essential to explore the underlying mechanism through biochemical assay in development of resistance to support the conventional method (Brogdon et al., 1997). Insect populations survive the effect of toxic insecticidal compounds by different physiological mechanisms, including reduced target site sensitivity such as kdr (Knock down resistance) mutation and elevated detoxifying enzyme production (Martinez-Torrres et al., 1998). This kdr mechanism confers resistance to pyrethroid insecticides and DDT through point mutations in the voltage-gated sodium channel gene (Williamson et al., 1996). The resulting single amino acid changes in the domain II region of the sodium channel reduce the sensitivity of the insect nervous system to these compounds. Some kdr allele has predominantly been found in resistant *An. gambiae* s. s (Chandre et al., 2000; Awolola et al., 2003). and several reports identified the presence of kdr in the sibling species, *Anopheles arabiensis* (Verhaeghen et al., 2006; Diabate et al., 2004; Stump et al., 2004). However, in metabolic resistance mechanism there are four important detoxifying enzymes i.e. AChE, ETS, Cyt p450 and GST. Measuring the activity of these enzymes in field populations is an important step in monitoring insecticide resistance mechanism (Isabel et al., 2012). It is reported that in most cases, where DDT is only used to control vector density, resistance arises due to elevated GST(Rodriguez et al., 2007). However, in case of cross resistance to pyrethroids, multiple enzymes involvement was also reported. GST is a well known insecticidal resistant indicator and was found elevated in DDT resistant strains of field collected *Anopheles gambi* (malaria vector) in Cameroon (Tene et al., 2013). In the Genetic analysis of mosquitoes GST mRNA levels of aggst3-2 were approximately 5-fold higher in DDT resistant strain than in the susceptible strain and demonstrated that recombinant AgGST3-2 had very high DDT dehydrochlorinase activity (Ranson et al., 2001). In *Aedes aegypti*, mutation in trans-acting repressor causes the over expression of GST, which ultimately causes resistant development of these species (Mendoza et al., 2009). However, sand fly population from areas using Deltamethrin impregnated bed-nets showed no resistance against it (Mukhopadhyay et al., 1992). Hence, presence of cross resistance can be neglected. Hence, this study was formulated to see the involvement of metabolic mechanism of resistance against DDT in *P. argentipes.*

MATERIALS AND METHODS

Collection and Rearing of Sand Flies

Wild Sand flies resistant to DDT, established from collections of indoor resting adult *P. argentipes* from cattle sheds were collected on three consecutive mornings between 6:00 A.M. to 8:00 A.M. during March-April 2012 from three endemic villages; Baizalpur of Sonepur/Saran District (Latitude 26.5376^0 N & Longitude 81.3328^0 E), Chandi of Nalanda District (Latitude $25^0$19'0" N & Longitude $85^0$24'0" E) and Khusroopur of Patna District (Latitude 25.4667^0 N & Longitude 85.3833^0 E) of Bihar, India. The sand flies collected were reared in insectary of Rajendra Memorial Research Institute of Medical Sciences, Agamkuan Patna, Bihar, India following the protocol of (Hassan et al., 2010) with slight modifications to develop the F1 progeny out of which one sub sample was stored at -80^0C for biochemical assay while the other was used for insecticide bio-assay test.

Bio-Assay Test

To determine the current status of insecticide resistance in the population bio-assays were performed on sub sample of F1 generation with female *P. argentipes* (n = 20) following the protocol of WHOPES (2006) with 4% DDT & 0.5% Deltamethrin impregnated paper.

Total Protein Assay

Individual *P. argentipes* (n=300) from F1 generation were homogenized in 200 µl of PBS. Total protein concentration of sand flies for biochemical assay was analyzed by Lawry's method (1951).

Glutathione-S-Transferase (GST) Activity

Single *P. argentipes* (10 µl) Homogenate was mixed with 200µl of substrate (125µl of 63mM CDNB in 10ml methanol + 2.5ml of 10mM reduced glutathione in 2.5ml 0.1M phosphate buffer). The reaction rate was measured at 340 nm after 5 min using U. V spectrophotometer. An extinction co-efficient of $5.3mM^{-1}$(corrected for path length of 0.552cm) was used. Activity of enzyme was evaluated in µmol/min/mg protein. Enzyme activities were recorded as µmol product $min^{-1}mg^{-1}$protein.

Acetylcholinestrase (AChE) Activity

Crude homogenate (25 µl) in replicate were transferred to a Microtitre plate and 145 µl of Triton phosphate buffer (1 % v/v Triton X-100 in 0.1 M phosphate buffer pH 7.8) was added to each replicate. 10 µl of 0.01 M dithiobis 2-nitrobenzoic acid (DTNB) solution in 0.1 M phosphate buffer pH 7.0 and 25 µl of the substrate 0.01 M acetylcholine iodide (ASChi) (HiMedia) was added to one of the replicates to initiate the reaction. Also, 25 µl of ASChI containing 0.2% (v/v) of the inhibitor propoxur (0.1M) was added to the second replicate. The reaction of the enzyme was continuously measured at 405nm for 5min in an ELISA reader (Bio-Rad, USA). The inhibition percentage of AChE activity due to propoxur, as compared to uninhibited wells was calculated. The residual activity of more than 80% suggested homozygosity for an altered AChE whereas values between 60% and 80% suggested heterozygosity for sand flies (Surendran et al., 2005).

Non-Specific Esterase (ETS) Activity

Homogenate was prepared and transferred to separate wells in replicates (20 µl each) in a Microtitre plate. In one replicate, 200 µl α-napthyl acetate solutions (100 µl of 30mM alpha-napthyl acetate in acetone diluted in 10ml of 0.02M phosphate buffer pH 7.2) was added and 200 µl β-napthyl acetate (prepared as for α-napthyl acetate) was added to other replicate. The plate was incubated for 15 minutes at room temperature and then 50 µl of Fast Blue Stain solution (150mg Fast Blue in 15ml distilled water) and 35 µl of 5% sodium lauryl sulphate (SDS) were added to each well. In blank all reagents without sand fly homogenate was taken as negative control. Enzyme activity was recorded at 570 nm as an end point reading in an ELISA reader (Bio-Rad, USA). Ranges of concentration (2µg-500 µg) α and β-napthyl acetate (Himedia) solutions were used to setup standard curves to determine the concentration of reaction products α and β-napthyl acetate in µmol product $min^{-1}mg^{-1}$ protein.

Cytochrome p450 (Cyt p450) Monooxygenases

The homogenate (10 µl) was mixed with 80 µl of potassium phosphate buffer (pH 7.2). Further 200 µl of 6.3 mM tetramethyl benzidine (TMBZ) working solution (0.01 g TMBZ dissolved in 5 ml methanol and 15 ml of sodium acetate buffer pH 5.0) and 25 µl of 3 % (v/v) H_2O_2 solution were added in a Microtitre plate well. After two hours incubation at room temperature, the plate was read at 630 nm as an endpoint assay using an ELISA reader (Surendran et al., 2005). Values were compared with a standard curve of absorbance for known concentration of Cytochrome C and were recorded as equivalent units of Cytochrome p450 mg^{-1} protein, for correcting the known haem content of Cytochrome C and p450.

SDS-PAGE

The pools of the same sample which was used for the GST enzyme activity from tolerant & susceptible strains of *P. argentipes* were concentrated in single 1.5 ml tube separately and protease inhibitor was added. The protein concentration was determined by using 200 µl of protein reagent solution (GeNei^{tm} protein estimation kit), 10 µl of the homogenate was added to it and incubated at 65ºC for 30 minutes. Total protein was measured at 560 nm following Lawry's method (1951). Proteins were separated with equal amounts (26 µg) each from resistant and susceptible strain on 12% SDS-PAGE for 45 minutes at 120 V on vertical electrophoresis unit (Bio-Rad, USA). The gel was stained with coommasie brilliant blue and destained following Lowe Protocol (1979). The band intensity obtained by SDS-PAGE was quantified by Bio-Rad Quantity One software.

Elution and Quantification of GST

The protein band was eluted and renatured according to Hager with slight modification (Hager et al., 1980). Bands obtained on SDS-PAGE were cut and crushed with autoclaved small Teflon pestle (Kontes, K886001 size 19) in elution buffer. The protein was allowed to elute for 1 hr at 25 ºC with occasional agitation and pelleted the crumbled gel through centrifugation for 1 min. The SDS was removed by adding cold acetone (-20 ºC) and samples were allowed to precipitate. The tubes were then centrifuged for 10 min at 10,000 rpm and acetone supernatants poured off. Renaturation was done through the treatment of sample by dissolving it with 20 µl of 6 M guanidine-HCL in dilution buffer. The pellets were dissolved thoroughly and allowed to stand at room temperature for 20 min. The solution was then diluted 30-fold with dilution buffer and allowed to renature for 8 h at room temperature. The activity of renatured enzyme was checked as described in experimental procedure along with a whole protein lysate of DDT resistant strains of *P. argentipes* as positive control for enzymatic activity.

Western Blotting

Sand fly extracts (26 µg total protein) from same sample which was used in Biochemical assay were pooled down and were analysed by SDS-polyacrylamide gel electrophoresis (12% acrylamide running gel and 4% acrylamide stacking gel) and electroblotted onto PVDF membrane. The membrane was probed for two hours with an anti-GST antibody (Santacruz) at 1:1000 dilutions. Immunoreactive proteins were visualised using Western blotting kit (GE Healthcare). Intensity of bands obtained was quantified with Biorad Quantity One software.

RESULTS

Identification of *P. argentipes*

All female sand flies were identified under microscope morphologically as *P. argentipes* following Lewis Key (1978).

Bio- Assay Test

The mortality of *P. argentipes* against 4% DDT from each village was found to be 50%, 55% and 45% from Baizalpur, Chandi and Khusroopur villages respectively. In Deltamethrin bio-assay test of F1 generation sand flies, 100 % mortality was observed.

Biochemical Tests of Four Enzymes

Independent student t-test revealed no significant differences (P>0.05) in the level of ETS, Cyt p450 and AChE

between the F1 *P. argentipes* of the three different villages populations. However, the GST enzymes showed a significant difference between *P. argentipes* of the three populations (t=5.41; p<0.005). The measured specific activity of GST was found to be higher in total of 180 (male=80, female=100) individual out of 300 tested samples with median GST activity of whole sample being 1.64±0.05 µmol/min/mg, whereas, the GST activity for susceptible strain was observed to be lying between 0.34±0.07-0.42±0.06µmol/min/mg as similar to the established cut-off range for susceptible strain for *P. argentipes* by surendran et al., (2005) in Sri Lanka (Figure 1) suggesting its involvement in metabolic resistance against DDT.

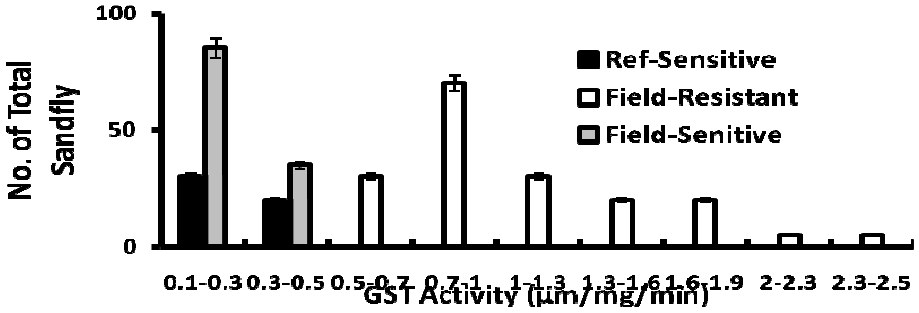

Figure 1: GST Activity in Resistant and Susceptible Strains of P. Argentipes

Similarly specific ETS activity was measured with cut-off (0.02±0.008 µmol/min/mg protein and 0.9±0.07 µmol/min/mg) and was found below the cutoff in all the samples. Furthermore, AChE residual activity was found less than 80% as suggested for non-altered AChE enzyme and Cyt p450 levels in all *P. argentipes* had values within susceptible range. However, out of 180 specimens with elevated GST only one specimen had high ETS activity i.e., 2.52 ± 0.5 µmol/min/mg proteins.

SDS PAGE

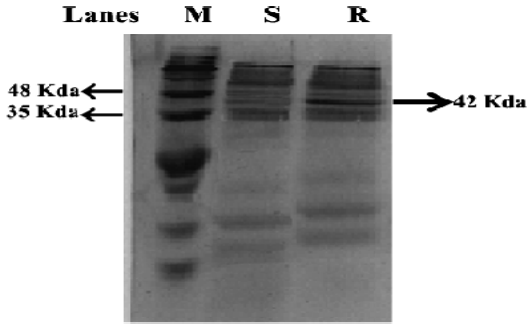

Figure 2: Whole Cell Protein Profiles of the Resistant and Susceptible Isolates on SDS-PAGE

Highly intense protein band at around 42 kDa was found in resistant versus sensitive isolates on 12% SDS-PAGE. The separated protein profile suggested that band at around 42 kDa was highly expressed in case of resistant isolates of *P. argentipes* in comparison to susceptible isolates (Figure 2).

Elution and Quantification of GST

On elution and renaturation of the protein in a highly intense band in SDS-PAGE the GST activity was found to be 0.85, 0.21 and 1.1 (µmol/min/mg) in resistant, susceptible strains and Whole cell lysate of resistant isolates respectively. Therefore, the highly expressed band of resistant isolates was detected as GST.

Western Blotting

A single band of approximately 42 kDa was detected and confirmed as GST in SDS-PAGE. The band was highly expressed in resistant isolates compared to the sensitive isolates (Figure 3).

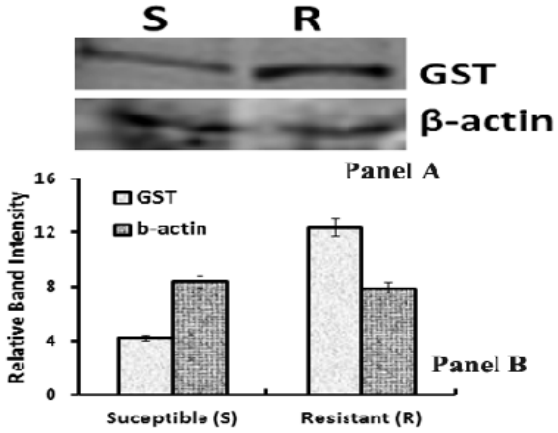

Figure 3: Western Blotting of Resistant and Susceptible Isolates

DISCUSSIONS

In the mechanism of development of resistance DDT is converted to 1,1-*bis*-(4-chlorophenyl)-2,2-dichloroethene (DDE) by the action of a number of Glutathion-S-transferase Clark et al., 1984). High frequencies of esterase and GST was found in organophosphorous and pyrethroid resistant *Aedes aegypti* in Latin America (Rodriguez et al., 2007). Reports also suggested the role of elevated GSTs level associated with DDT resistance in mosquitoes by detoxification of insecticide in laboratory condition (Ding et al., 2003; Mouches et al., 1987) supporting this biochemical assay results for organo-chlorine resistant strains. In a recent study, it was reported that single amino acid change L119F in an unregulated glutathione S-transferase gene, GSTe2, confers high levels of metabolic resistance to DDT in malaria vector *Anopheles funestus* (Riveron et al., 2014). In the present study, GST specific activity in 60% of population from three different villages lies above the susceptible range suggesting the elevation of GST enzyme in DDT resistance, similar to the results of Grant et al. (1989) for DDT resistance in mosquitoes. However, one specimen with elevated GST was found to have higher ETS enzyme activity which may be due to exposure to deltamethrin impregnated net exposure. This result was found to be in concordance with the previously reported susceptibility status of *P. argentipes* by WHO susceptibility test. The high intensity band of GST found in SDS PAGE and Western blot in resistant strains supported the high expression of GST in resistant *P. argentipes* population correlating to the biochemical assay. This study strongly suggests that the current development of DDT resistance in *P. argentipes* in some parts of Bihar region is partly due to elevated levels of GST enzyme (one of the important detoxifying enzymes), similar to the results found with a different DDT resistant strain of other Dipterans. Study of knock down resistance was avoided since there was no cross resistance to deltamethrin, as it arises only in cross resistance of DDT and other pyrethroids (WHO, 1998).

CONCLUSIONS

The present study provides preliminary biochemical evidence of GST enzyme elevation in DDT resistant *P. argentipes* population. Proteomic approach for studying resistance mechanism would be a better strategy since the genome sequence of *P. argentipes* is yet unavailable in the data base.

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Ethical Approval: There is no involvement of ethical clearance.

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